ZOOLOGICAL SCIENCE 8: 23–29 (1991)

Immunolocalization and *in vitro* Secretion of Hemolymph Lectin of the Pearl Oyster, *Pinctada fucata martensii*

Tohru Suzuki and Katsuyoshi Mori

Nutrition Division, National Research Institute of Aquaculture, Fishery Agency, Nansei, Mie, 516-01 Japan

ABSTRACT—Identification of the organ which secretes hemolymph lectin in the pearl oyster, *Pinctada fucata martensii,* was sought by means of immunocytochemistry and *in vitro* tissue culture. Apparent immunoreaction to anti-hemolymph lectin antiserum was detected from the mantle. The immunoreaction was localized in the granular cells under the mantle epithelium. When mantle explants were cultured, they secreted hemolymph lectin into the culture medium. These results suggest that the hemolymph lectin is localized at and secreted from the mantle.

INTRODUCTION

Hemolymph of various invertebrates shows hemagglutination activity toward vertebrate erythrocytes [1–3]. Hemolymph lectins responsible for such activity have been isolated from Mollusca, Arthropoda, Echinodermata and Prochordata, and some of their biochemical properties have been characterized [for review, 4].

In most cases, however, it is not well understood which organ produces hemolymph lectin. At present, the flesh fry, *Sarcophaga peregrina* is the only invertebrate whose hemolymph lectin producing organ has been experimentally confirmed [5, 6]. In *S. peregrina,* it is the fat bodies of the larvae that synthesize and excrete hemolymph lectin. By contrast, in the cockroach, *Leucophaea maderae* [7, 8] and pond snail, *Lymnaea stagnalis* [9], hemocytes may be the cells which produce lectin as judged from immunocytochemical assays.

A galactose-specific lectin was isolated from the hemolymph of the pearl oyster, *Pinctada fucata martensii* and mono-specific anti-hemolymph lectin antiserum was prepared [3]. Although immunocytochemical assays using this antiserum were applied to the hemocytes of this bivalve, the lectin could not be visualized in them [10]. Therefore, it was concluded that hemocytes do not possess hemolymph lectin and inferred that it is synthesized in some other organ.

This report aims to determine the organ which secretes the lectin. We attempted to localize the lectin in the pearl oyster and then evaluated the lectin secreting ability of the immunoreactive organ.

Accepted August 16, 1990
Received April 9, 1990

MATERIALS AND METHODS

Animals

Pearl oysters '*Pinctada fucata martensii*' cultured at the National Research Institute of Aquaculture, Nansei, Mie, Japan were used in this study. The animals were 10 - 13 cm in shell length. Examinations were performed in May, 1989.

Antiserum

Rabbit anti-hemolymph lectin antiserum was prepared according to previous methods [3].

Indirect immunofluorescence

Five pearl oysters were dissected and fixed in Bouin's fixative for 6 hr at 4°C. Fixed samples were washed three times in 0.01 M phosphate buffer, pH 7.2, containing 0.15 M NaCl (PBS) for 1 hr, dehydrated through a series of graded alcohols and embedded in paraffin. Sections of 7 μm were prepared and mounted on slides. After removal of the paraffin, sections were incubated

with 1% bovine serum albumin in PBS and then with anti-hemolymph lectin antiserum (1 : 100 dilution with PBS) or normal rabbit serum for 30 min. After three washes in PBS, they were incubated for 30 min with fluorescein isothiocyanate (FITC)-conjugated swine anti-rabbit immunoglobulins (Ig) (Dakopatts Co.) (1:100 dilution) and washed twice in PBS.

Electron microscopy

The dorsal mantle was fixed in Karnovsky's fixative containing 8% sucrose for 1 hr at 4°C. The sample was washed twice in 0.1 M phosphate buffer, pH 7.2 (PB), containing 8% sucrose and then post-fixed in 1% osmic acid in PB. After washing in PB, the sample was dehydrated through a series of graded alcohols and embedded in TAAB 812 resin. Semi-thin and ultra-thin sections were cut by using a Porter-blum MT2-B ultramicrotome. Ultra-thin sections were stained with uranyl acetate and lead citrate and examined by a JEOL-1200EX electron microscope. Semi-thin sections were stained with methylene blue-Azur II solution.

Immuno-electron microscopy

The dorsal mantle was fixed in Zamboni's fixative for 2 hr at 4°C. The fixed sample was washed twice in PBS, and then cut into tissue sections (30 μm thick) using a microslicer DSK-1000 (Dosaka Co.). Sections were mounted on slides, dried and incubated with 1% bovine serum albumin in PBS overnight. They were then incubated with anti-hemolymph lectin antiserum (1 : 100 dilution with PBS) or normal rabbit serum for 12 hr at 4°C. After incubation, they were washed in PBS and incubated with horseradish peroxidase (HRP)-conjugated goat antibody ($(Fab)_2$ fragment) against rabbit Ig (Janssen Chimica) (1 : 20 dilution) for 12 hr at 4°C. They were rinsed twice in PBS, fixed in 1% glutaraldehyde in PB for 1hr and incubated for 15 min in diaminobenzidine (DAB)-H_2O_2 medium for peroxidase. They were post-osmicated for 60 min, then dehydrated and embedded in resin. Ultra-thin sections were examined without electron staining.

Tissue culture

After removing one of the shells, a small piece of mantle explant (4×4 mm square and 1 mm in thickness) was cut from the dorsal region of six animals. A piece of digestive diverticula explant was also obtained. Explants were washed five times in a balanced salt solution for marine molluscs (MMBSS) which was prepared as described by Machii and Wada [11]. After pre-incubation in MMBSS for 2 hr, each explant was incubated in 400 μl of new MMBSS containing antibiotics (penicillin and kanamycin) for 20 hr at 20°C. After incubation, the hemagglutination activity of the culture medium was determined using horse erythrocytes according to a method previously reported [3].

Hemagglutination blocking test

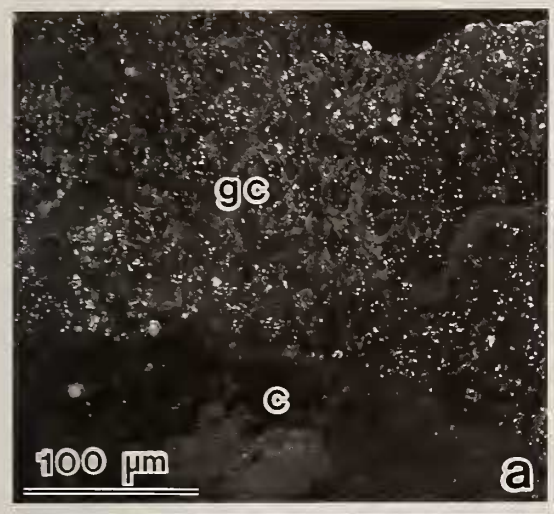
Culture mediums incubated with mantle explants were collected and prepared to give a hemagglutination titer of 2^3. To the medium was added one of the following (final concentration is indicated in parentheses): MMBSS (1%), anti-hemolymph lectin antiserum (1%), normal rabbit serum (1%), galactose (20 mM) or glucose (20 mM). After the solutions stood for 1 hr at room temperature, their remaining hemagglutination activity was determined.

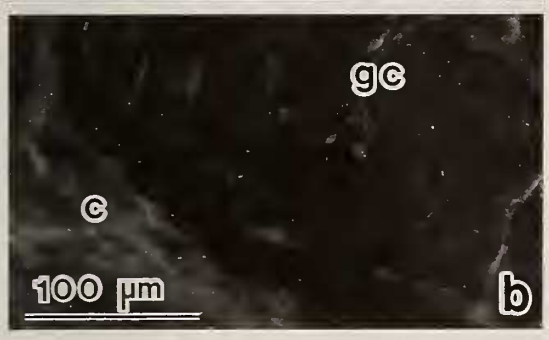
RESULTS

Immunofluorescence

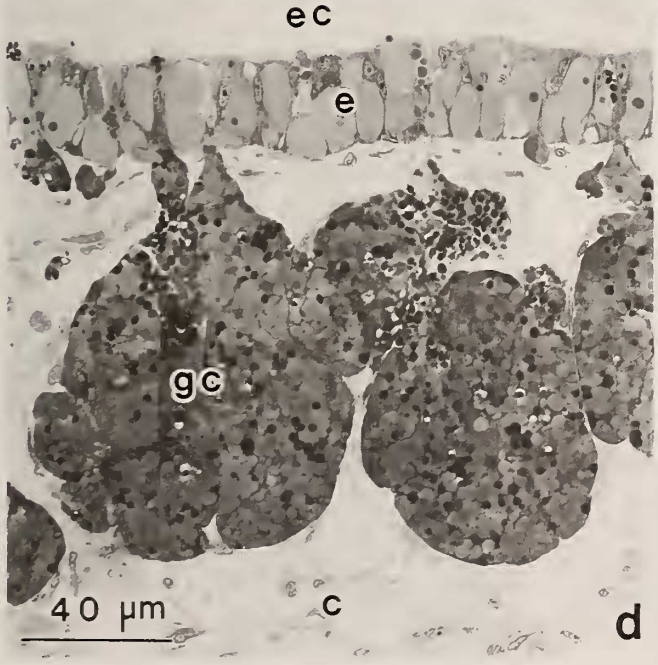
In indirect immunofluorescence using anti-hemolymph lectin antiserum, immunoreaction was

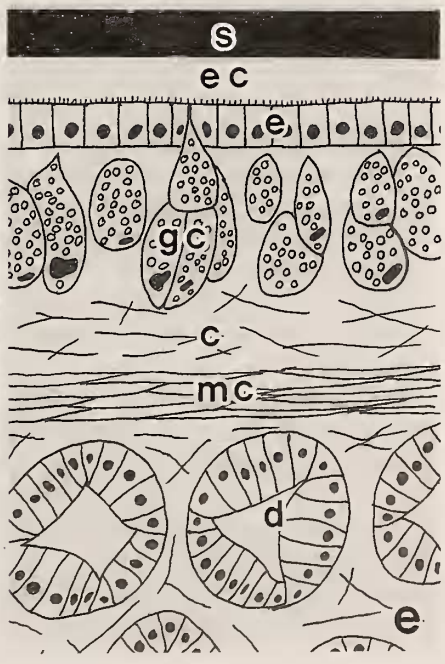
FIG. 1. Immunolocalization of hemolymph lectin in the pearl oyster. **a.** Indirect immunofluorescence applied to the mantle using anti-hemolymph lectin antiserum, showing that the lectin is localized in the granular cell layer of the mantle. **b.** Control staining of the mantle using normal rabbit serum. **c.** Indirect immunofluorescence using anti-hemolymph lectin antiserum, showing that connective tissue, muscle cells and digestive diverticula are not immunoreactive, whereas the granular cell layer of the mantle is reactive. **d.** Semi-thin section of dorsal mantle. **e.** Schematic representation of the mantle and digestive diverticula histology. c, connective tissue; d, digestive diverticula; e, epithelium; ec, extrapallial cavity; gc, granular cell; mc, muscle cell; s, shell.


gc
c
100 µm
a


gc
c
100 µm
b


gc
c
mc
d
100 µm
c


ec
e
gc
c
40 µm
d


s
ec
e
gc
c
mc
d
e

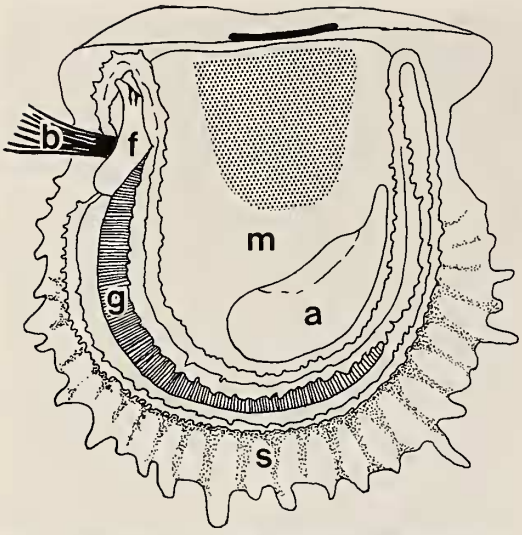


Fig. 2. Distribution of the granular cells immunoreactive to anti-hemolymph lectin antiserum. The granular cells are distributed in the mantle of the central and dorsal regions of the body (dotted). a, adductor muscle; b, byssus; f, foot; g, gill; m, mantle; s, shell.

visualized as a granular fluorescent staining in the mantle (Fig. 1a). In particular, the reaction was observed in the mantle of the central and dorsal regions of the body (Fig. 2). When normal rabbit serum was used instead of the antiserum, the mantle did not show fluorescent staining. (Fig. 1b).

The mantle possessed a single layer of epithelial cells, the free surface of which was exposed to the fluid in the extrapallial cavity (Fig. 1d, e). Under the epithelium of the central and dorsal mantle, a granular cell layer was present. Loose connective tissue was observed between the granular cell layer and muscle cell layer which enveloped the digestive diverticula.

The granular fluorescent staining to anti-hemolymph lectin antiserum was localized in the granular cells of mantle (Fig. 1a, c). Connective tissue, muscle cells and digestive diverticula were not immunoreactive (Fig. 1c). In addition, no immunoreaction was detected from the stomach, intestine, heart, kidney, gonad and gill (data not shown). Such a localization pattern of lectin was common to the five animals examined.

Ultrastructure of granular cells

The ultrastructure of the granular cells under the mantle epithelium is shown in Figure 3. The granular cells were oval- or pear-shaped in transverse section and the major axis was 20–100 μm. The nucleus was peripherally located in the cells. Cytoplasmic granules which characterized the cells showed various electron density. Their diameter was 0.7–4.0 μm. In large, perhaps mature cells, the granules were densely packed in the cytoplasm, compressing the nuclei (Fig. 3a). Other organelles, such as the endoplasmic reticulum, Golgi apparatus and mitochondria were not well developed. In contrast, small, possibly immature cells possessed a developed endoplasmic reticulum, Golgi apparatus and mitochondria (Fig. 3b).

Immuno-electron microscopy

In immuno-electron microscopy using an enzyme-labeled antibody method, granular staining for anti-hemolymph lectin antiserum was also observed from granular cells of the mantle as in the case of indirect immunofluorescence (Fig. 4 a, b). A part of the cytoplasmic granules were immunoreactive to the antiserum (Fig. 4c). The positive granules were 0.7–1.5 μm in diameter and relatively small compared with other granules in the cells.

Secretion of lectin from the mantle

Since the mantle was immunoreactive to the anti-hemolymph lectin antiserum, we evaluated its lectin secreting ability by culturing mantle explants *in vitro*. Digestive diverticula explants were also cultured as control assays. Hemagglutination activity of the culture medium at 20 hr of incubation is indicated in Table 1. The medium of the mantle explant of each animal examined showed hemagglutination activity, while that of the digestive diverticula did not.

A hemagglutination blocking test was performed to determine whether the hemagglutination activity of the culture medium was caused by the hemolymph lectin (Table 2). Both anti-hemolymph lectin antiserum and galactose, a hapten sugar of the lectin [3], perfectly inhibited hemagglutination activity of the culture medium of

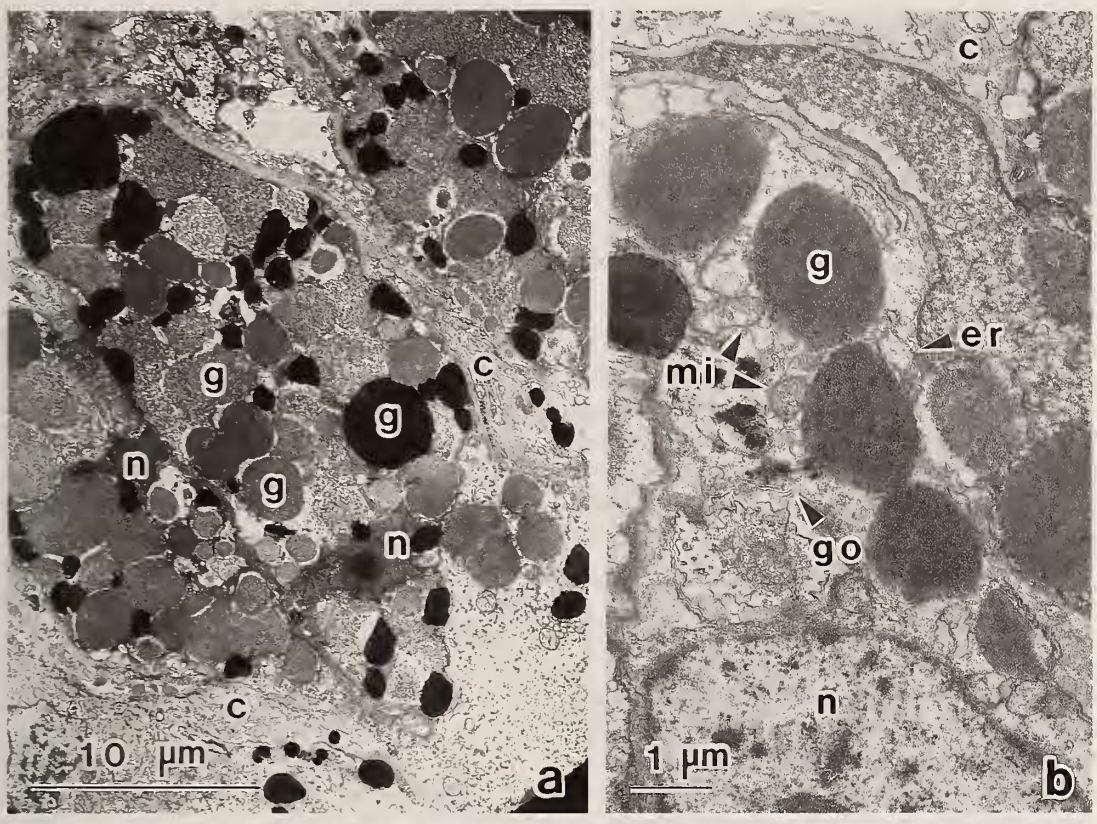


FIG. 3. Ultrastructure of the granular cells of the mantle. **a**. Large-sized granular cells. Note the densely packed granules of various size and electron density. **b**. Small-sized granular cell. Note the developed endoplasmic reticulum and Golgi apparatus. c, connective tissue; er, endoplasmic reticulum; g, granule; go, Golgi apparatus; mi, mitochondria; n, nucleus.

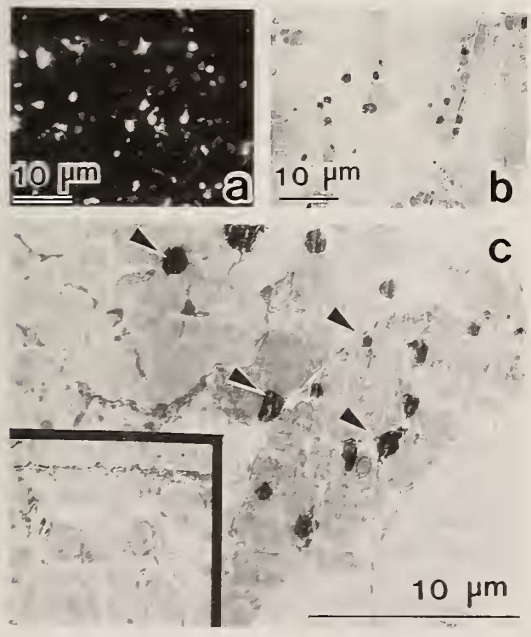


TABLE 1. Hemagglutination activity in culture medium at 20 hr of incubation with mantle and digestive diverticula explants (details in text)

Animals	Explant	
	Mantle	Digestive diverticula
a	2^4	0
b	2^3	0
c	2^3	0
d	2^2	0
e	2^2	0
f	2^2	0

FIG. 4. Immunolocalization of hemolymph lectin in the granular cells of the mantle. **a**. Indirect immunofluorescence using anti-hemolymph lectin antiserum. **b**. Immuno-electron microscopy at the same magnification as **a**. **c**. Higher magnification of a granular cell. Note that small granules exhibit immunoreaction (arrow heads). Inset is a control staining using normal rabbit serum.

TABLE 2. Blocking of hemagglutination activity secreted from mantle explants

Additives	Concentration	Hemagglutination titer
Balanced salt solution (MMBSS)	1%	2^3
Anti-hemolymph lectin antiserum	1%	0
Normal rabbit serum	1%	2^3
Galactose	20 mM	0
Glucose	20 mM	2^3

mantle explants. When normal rabbit serum and glucose, which does not affect the activity of the lectin, were added to the medium, hemagglutination activity did not change. These results indicate that galactose-specific lectin, which is immunologically identical with that of hemolymph, was secreted by the mantle explants into the culture medium.

DISCUSSION

Hemolymph of the pearl oyster shows strong hemagglutination activity toward horse erythrocytes, and a galactose-specific lectin responsible for this activity was isolated by three step chromatographies [3]. As to the physiological function of lectin, the previous report [10] suggested that it is a humaral defense factor functioning in the recognition of foreign particles. However, which organ secreted the lectin remained unknown.

In this paper, therefore, the secreting organ of the lectin was sought by means of immunocytochemistry and culture experiments. The following two important results were obtained. First, granular cells under the mantle epithelium of the central and dorsal regions concentrated the lectin in their cytoplasmic granules. Second, mantle explants secreted the lectin into culture medium. From these results, we suggest that the mantle contains and secretes the lectin into the hemolymph.

Identification of the synthesizing organ of hemolymph lectin has been sought in several invertebrates. In flesh fry (*S. peregrina*) larvae, transcription of the lectin gene is activated by injury to the body wall [6], after which the amount of lectin increases in the fat body and then in the hemolymph [5]. Thus, the hemolymph lectin of this insect is surely synthesized by the fat body. As far as we know, pearl oysters lack the organ which is functionally equivalent to the insect fat body.

In the cockroach, *L. maderae* [7, 8] and pond snail, *L. stagnalis* [9], hemocytes are suggested to be the producing cells of hemolymph lectin. This is based on the fact that their cytoplasm is immunoreactive to anti-hemolymph lectin antiserum. In contrast, pearl oyster hemocytes do not contain hemolymph lectin [10]. Such a disagreement as to the existence of hemolymph lectin in hemocytes may occur due to differences in species or the presence of heterogeneous lectins in organisms.

REFERENCES

1 Boyd, W. C., Brown, R. and Boyd, L. G. (1966) Agglutinins for human erythrocytes in mollusks. J. Immunol., **96**: 301–303.

2 Brown, R., Almodovar, L. R., Bhatia, H. M. and Boyd, W. C. (1968) Blod group specific agglutinins in invertebrates. J. Immunol., **100**: 214–216.

3 Suzuki, T. and Mori, K. (1989) A galactose-specific lectin from the hemolymph of the pearl oyster, *Pinctada fucata martensii*. Comp. Biochem. Physiol., **92B**: 455–462.

4 Olafsen, J. A. (1986) Invertebrate lectins-biochemical heterogeneity as a possible key to their biological function. In "Immunity in Invertebrates". Ed. by M. Brehelin, Springer-Verlag, Berlin/Heidelberg/New York/Tokyo. pp. 94–111.

5 Komano, H., Nozawa, R., Mizuno, D. and Natori, S. (1983) Measurement of *Sarcophaga peregrina* lectin under various physiological conditions by radioimmunoassay. J. Biol. Chem., **258**: 2143–2147.

6 Takahashi, H., Komano, H., Kawaguchi, N., Kitamura, N., Nakanishi, S. and Natori, S. (1985) Cloning and sequencing of cDNA of *Sarcophaga peregrina* humoral lectin induced on injury of the body wall. J. Biol. Chem., **260**: 12228–12233.

7 Amirante, G. A. (1976) Production of heteroagglutinins in haemocytes of *Leucophaea maderae* L. Experientia, **32**: 526–528.

8 Amirante, G. A. and Mazzalai, F. G. (1978) Synthesis and localization of hemagglutinins in hemocytes

of the cockroach *Leucophaea maderae* L. Dev. Comp. Immunol., **2**: 735–740.

9 Van der Knnap, W. P. W., Boerrigter-Barendsen, L. H., Van den Hoeven, D. S. P. and Sminia, T. (1981) Immunocytochemical demonstration of a humoral defense factor in blood cells (Amoebocytes) of the pond snail, *Lymnaea stagnalis*. Cell Tissue Res., **219**: 291–296.

10 Suzuki, T. and Mori, K. (1990) Hemolymph lectin of the pearl oyster, *Pinctada fucata martensii*; a possible non-self recognition system. Dev. Comp. Immunol., **14**: 161–173.

11 Machii, A. and Wada, K. T. (1989) Some marine invertebrates tissue culture. In "Invertebrate Cell System Application. Vol. II". Ed. by J. Mitsuhashi, CRC Press, Boca Raton. pp. 225–233.